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Molecular Genotyping of HIV-1 in 61 Patients With AIDS From Lomé, Togo

F.J. Davies,[1] O. d'Almeida,[2] E. Timmers,[1] J. d'Almeida,[2] M. Fasken,[1] K. Bassabi,[3] H. Lee,[1] and J.-P. Allain[1]*

[1]*Division of Transfusion Medicine, Department of Haematology, University of Cambridge, England*
[2]*Centre Régional de Diagnostic Médical, Lomé, Togo*
[3]*Comité National de Lutte Contre le SIDA, Lomé, Togo*

To study the distribution of HIV types and genotypes, in Lomé, Togo, a random population of patients who met the clinical criteria of the Bangui definition of AIDS and were positive with two independent screening assays for antibodies to HIV-1 group M, HIV-2, and HIV-1 group O was selected. HIV RNA from serum samples was reverse-transcribed and amplified with degenerate primers annealing to conserved regions of the HIV-1, HIV-2, and HIV-O *gag* gene. Amplicons were directly sequenced using an automated sequencer. A 262–271-bp (strain-dependent) fragment of the *gag* gene from each patient was phylogenetically analyzed and compared to the corresponding *gag* sequences of published HIV-1 sequences of known African genotypes. Genotype A was found in 48 of 60 patient amplicons (80%), subdivided into two clusters. Ten patients (16.7%) were HIV-1 genotype G; one was genotype D and one genotype H. HIV-1 genotype B was not found. Amplicons from two patients contained sequence ambiguities, requiring cloning and sequencing of the *gag* insert. One patient (T52) was apparently infected with HIV-1 genotypes A and G; whereas HIV-1 from patient T139 was of genotype A, with 2/10 clones having a three-codon insertion at nucleotide position 1142 of the *gag* gene. HIV-1 genotype A is dominant in Togo; genotype G is frequent and genotype B has not been found. ***J. Med. Virol. 57:25–30, 1999.***

KEY WORDS: HIV-1 subtype; phylogenetic analysis; *gag;* molecular epidemiology

INTRODUCTION

The global spread of HIV is resulting in the continuous occurrence of new genetic variants of the virus. HIV-1 variants in different geographic regions have been phylogenetically classified into distinct genotypes A-I and O. Many countries of sub-Saharan Africa face a public health crisis caused by the extensive spread of HIV infection. Numerous studies have been conducted on the distribution of the HIV-1 genotypes which, with the exception of genotype I, have all been found on the African continent. Three main genotypes are prevalent: A, C, and D. In all parts of sub-Saharan Africa, except Southern Africa, genotype A is dominant [Potts et al., 1993; Janssens et al., 1994c; Nkengasong et al., 1994]. Genotypes A and D are frequently found in eastern Africa [Janssens et al., 1994b; World Health Organization, 1994]; genotype C is dominant in southern Africa and Ethiopia [Sherefa et al., 1994; Becker et al., 1995; van Harmelen et al., 1997].

The greatest genotypic diversity is observed in central African countries [Murphy et al., 1993; Nkengasong et al., 1994; Delaporte et al., 1996], where, even in limited studies, as many as seven genotypes were found [Janssens et al., 1997]. The more distant genotype group O HIV-1 was found initially in Cameroon [Gurtler et al., 1994] and has since been identified in neighboring countries such as Nigeria [Peeters et al., 1997], Gabon [Delaporte et al., 1996], and Benin [Heyndrickx et al., 1996a]. The distribution of HIV-1 genotypes in the west African country of Togo has not yet been described. Since Togo is close to the divide between the greatest HIV-1 diversity of Central Africa and the fringes of the eastern spread of HIV-2, it is of interest to examine the type and genotype diversity of HIV in this country.

In view of the extreme variability of the envelope proteins, classification of HIV genotypes based on serology is unsuitable. Phylogenetic classification is frequently based on nucleotide sequence differences in the highly variable V3 region of the gp120 envelope gene. However, various regions of the *gag* gene, including a

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*Correspondence to: Dr. J.-P. Allain, Division of Transfusion Medicine, University of Cambridge, EABC, Long Road, Cambridge CB2 2PT, England.

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short segment coding for the *gag* p7/p9 protein, have been successfully used for phylogenetic analysis [Salminen et al., 1993]. In this study, sequences of a short segment of the *gag* gene were compared in 61 randomly selected patients with AIDS from Lomé, Togo.

MATERIALS AND METHODS

HIV-1–Positive Plasma Samples

Plasma samples were obtained from 61 random patients with clinical AIDS according to the Bangui definition [World Health Organization, 1986] from private clinics and hospitals in the capital of Togo, Lomé. The samples were tested for antibodies to HIV at the Centre Régional de Diagnostic Médical de Lomé and found positive by two commercial enzyme immunoassays, the Genetic Systems HIV-1/HIV-2 EIA (Sanofi Diagnostics Pasteur, France) and Enzymun-Test Anti-HIV1+2+Subtype O (Boehringer Mannheim, Germany). Assays were carried out according to the manufacturer's instructions.

Nucleic Acid Isolation and Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR)

Nucleic acid was extracted from 140 μl of plasma using the QIAamp Viral RNA extraction kit (Qiagen, U.K.). First-strand cDNA was synthesized using MuLV reverse transcriptase (RT) and random hexanucleotide primers. Reverse transcription was performed in 20 μl containing 10-mM Tris-HCl, pH 8.3, 50-mM KCl, 5-mM $MgCl_2$, 1 mM each dNTP, 3.2-μg random primer (Boehringer Mannheim), 20 units of RNase Inhibitor (Perkin-Elmer, Norwalk, CT), and 50 units of MuLV RT (Perkin Elmer). The reaction was incubated for 1 min at 25°C, 60 min at 42°C, 5 min at 99°C, and then cooled to 4°C.

The HIV cDNA was amplified using degenerate primers: GAG2FWD (5′-RGAYATAARRCARGGRCCAAA; nt position 842–862) and GAG2REV (5′-CTTKCCACAYTTCCARCARCCC; nt position 1239–1217) for the *gag* segment, and env27f and menv19r [Brennan et al., 1997] for the *env* segment. Five μl of cDNA was amplified by PCR in 50 μl containing 10-mM Tris-HCl, pH 8.3, 50-mM KCl, 2–2.5-mM $MgCl_2$, 200 μM each dNTP, 0.5–0.6 μM each primer, and 1.25 units of AmpliTaq Gold (Perkin Elmer). Amplifications were performed in a GeneAmp PCR System 2400 or 9600 by denaturation for 9 min at 94°C and then 45 cycles consisting of 30 sec at 94°C, 60 sec at 56°C for *gag* or 30 sec at 50°C for *env,* 60 sec at 72°C, and an incubation for 7 min at 72°C after cycling. All *env* segments were amplified by a single round of PCR except sample T52, which required nested PCR as described using the outer primers JH38 and JH41 [Brennan et al., 1997]. After electrophoresis, the DNA was visualized by ethidium bromide.

Cloning

Amplification products were isolated by electrophoresis; the PCR product was excised and purified using the QIAquick Gel Extraction Kit (Qiagen). Purified amplification products were cloned into pCR2.1-TOPO TA cloning vector (Invitrogen BV, the Netherlands). Plasmid DNA was extracted from white transformants using the Wizard Plus Minipreps DNA Purification System (Promega, Madison, WI). Plasmids were screened for the presence of the insert prior to sequencing.

DNA Sequencing

Nucleotide sequences were determined using the Thermo Sequenase dye terminator cycle sequencing premix kit (Amersham Life Science, U.K.). Sequence was obtained from both strands of the amplicon, primed with either the forward or the reverse PCR primer or from the cloned PCR product using the M13 forward or reverse sequencing primer. Electrophoresis and data collection were performed using an ABI 373 DNA sequencer (PE Applied Biosystems, CA).

Sequence Alignments and Pairwise Comparison

The DNA sequence data obtained from the *gag* region was edited and assembled using MacVector 6.0.1 (Oxford Molecular, U.K.) and Genetics Computer Group (GCG) (Wisconsin Package Version 9.1, Madison, WI) sequence analysis software. Multiple alignments were performed using CLUSTAL W [Thompson et al., 1994], and the alignment was optimized manually using SeqLab (GCG) to ensure that insertion or deletions occurred in groups of three nucleotides in order to preserve the amino acid coding frame.

Phylogenetic Analysis

Phylogenetic analysis was performed using the PHYLIP (Phylogeny Inference Package, version 3.5c) [Felsenstein, 1993] programs DNADIST, DNAPARS, SEQBOOT, NEIGHBOR, CONSENSE, and DRAWTREE. Trees were outgroup-rooted using group O HIV-1 sequences (ANT70, MVP5180, accession number: L20587, L20571) in the alignment. Distance matrices were generated with DNADIST using Kimura 2 parameter (K2P) model or maximum likelihood (ML). Statistical significance was tested by bootstrapping using SEQBOOT to produce bootstrapped datasets ($n = 100$). Trees were constructed from the distance matrices using the neighbor-joining (NEIGHBOR) method and from the resulting data a consensus tree was calculated using CONSENSE. Trees were visualized using DRAWTREE and TreeView 1.4 [Page, 1996].

Published HIV-1 *gag* region sequences were used as a reference for the various genotypes in the phylogenetic analysis. Genotype A: U455, VI59, VI354, LBV23-10, TN243 (accession number: M62320, L11795, L11790, L11777, L03702); B: BZ167, TB132, JRCSF, OYI (accession number: L11752, L03697, K02013, M38429, M26727); C: UG268, SM145, ZAM18, ZAM20, DJ259, VI313 (accession number: L11799, L11803, L03705, L03707, L11764, L11787); D: ELI, NDK, VI205, UG274, VI203 (accession number: K03454, M27323, L11785, L11801, L11784); F: VI174, VI69, BZ162, VI325 (accession number:L11782, L11796,

TABLE I. Common Symptoms of Patients Diagnosed in Lomé, Togo

Clinical elements	Kaposi's sarcoma	Cryptococcal meningitis	Weight loss	Chronic diarrhea	Fever	Polyadenopathies	Recurrent HerpesZ.	Oropharyngeal candidiasis	Cough
Number	11	0	44	39	36	2	5	17	6
% of total	18	0	72.1	63.9	59	3.3	8.2	27.9	9.8

L11751, L11789); G: LBV217, VI191 (accession number: L11778, L11783); H: VI525, VI557 (accession number: L11792, U09666), and two hybrid sequences K124 and MAL (accession number: L11769, K03456). Phylogenetic consensus trees constructed using the entire *gag* region were compared with those constructed with only the 262–271-bp fragment equivalent to the RT-PCR fragment, and were found to be largely congruent, thus validating the use of this region for HIV genotyping by phylogenetic analysis.

RESULTS

The sample population of 61 individuals consisted of 18 females and 43 males, with an average age of 32.7 and 36.4 years, respectively. All patients included in the study met the clinical criteria for AIDS according to the Bangui definition for AIDS in sub-Sahara and all sera were strongly positive with two screening assays for antibodies against HIV. These clinical and laboratory criteria were considered sufficient for the diagnosis of AIDS in all patients [World Health Organization, 1994]. The CD4 cell level was determined in five patients, and ranged from 71 to 769 CD4 equivalents/μl (mean 368/μl). As shown in Table I, weight loss, chronic diarrhea, and prolonged fever were the most common symptoms, with oropharyngeal candidiasis and Kaposi's sarcoma present in 27.9% and 18% of the patients, respectively.

From extracted viral RNA, a fragment of approximately 410 bp of the *gag* region was amplified using RT-PCR. The nucleotide sequence of 271 bp of this fragment was determined by direct automated sequencing of the amplicon. HIV sequences corresponding to nucleotides 878–1137 (isolate LBV23-10, L11779 [Louwagie et al., 1993]) were obtained from every one of the 61 patients tested and aligned for comparison.

In order to validate the use the 271-bp *gag* sequence for determining HIV genotypes, we established the statistical confidence of phylogenetic trees constructed from the alignment of the equivalent sequence fragment of 31 published sequences of HIV-1 genotypes A-H and O. In a consensus tree, the sequences are clustered according to their genotype as identified by Myers et al. [1995], (http://hiv-web.lanl.gov) (Fig. 1). The use of the 271-bp fragment gave similar results to consensus trees based on the entire *gag* region, with the exception of genotypes B and D, which give lower boot strap values of around 50%.

Phylogenetic analysis of the 60 partial *gag* sequences from Togolese patients, together with reference sequences, is shown in Figure 2. Forty-eight patient sequences (80%) clustered with representative genotype A isolates. These 48 sequences tend to form two distinct branches, with some sequences being more related to each other than to published sequences from other African countries. Ten sequences (16.7%) clustered with reference genotype G, one sequence with genotype D, and one with genotype H. No sequences related to genotype B were observed.

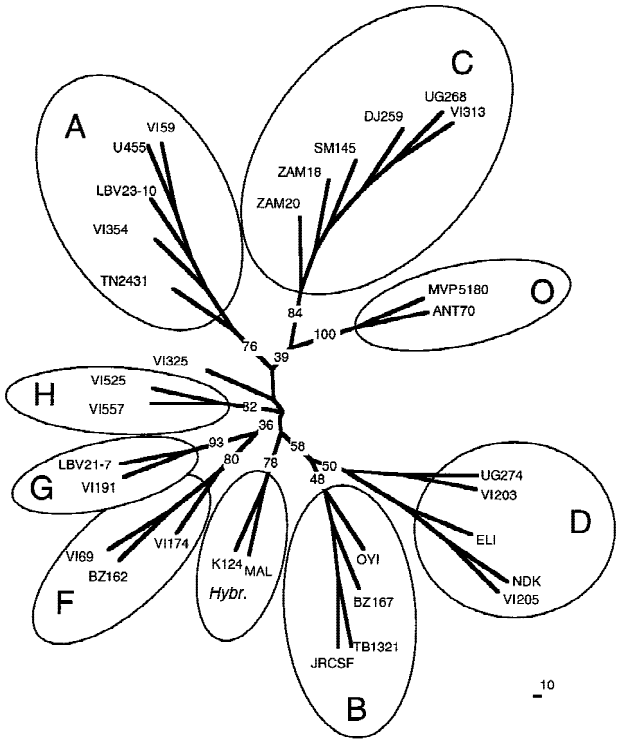

Fig. 1. Phylogenetic relationship between HIV-1 reference sequences comparing 271 bp (nt 878–1137) of the *gag* region. Multiple nucleotide sequence alignments were resampled by bootstrapping using 100 data sets and the PHYLIP software package.

Among the 61 patients, direct sequencing of the gag amplicon revealed unresolved ambiguities in two sequences. To resolve these ambiguities, the 400-bp amplicon from patients T52 and T139 were cloned and the nucleotide sequence of the partial *gag* insert of 12 and 10 recombinants, respectively, were determined. Phylogenetic analysis of the cloned sequences of patient T52 identified 10 sequences clustering with reference sequences of A genotypes and two sequences with genotype G (Fig. 3). Phylogenetic analysis of 10 clones from the amplicon of sample T139 indicated that all sequences were of genotype A, which clustered in 98 out of 100 bootstraps. This indicates that the variants originated from the same HIV quasispecies. Two of 10 sequences contained a three-codon insertion at nucleotide position 1146 (AGGGGGTCA), with eight nucleo-

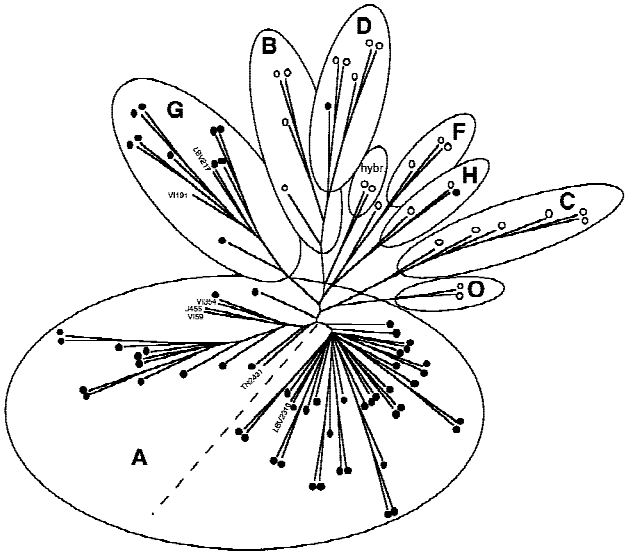

Fig. 2. Phylogenetic relationship between HIV-1 *gag* reference sequences and 60 patient sequences (sample T52 omitted in this tree) obtained from Togo. Open circles denote reference sequences (same as in Fig. 1) and closed circles indicate the unknown samples from Togo. A dashed line separates two branches within genotype A.

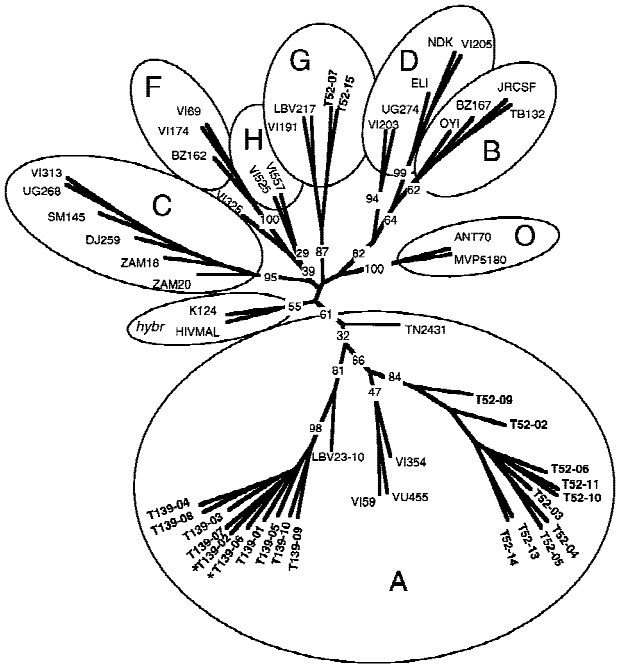

Fig. 3. Phylogenetic analysis of cloned HIV-1 *gag* sequences analogous to those in Figure 2. The distribution of the clones clearly indicates that the homologous sequences in T139 originated from the same quasispecies variant by an insertion event (asterisk indicates sequence with insertions), whereas patient T52 shows infection with both genotype A and genotype G of HIV-1.

tides of this insertion identical to the preceding eight nucleotides, suggesting a duplication event.

In order to confirm genotyping in the *gag* region, a stretch of 435 nucleotides of the *env* region was sequenced and analyzed in selected samples. The *env*-based genotypes of three randomly selected samples of genotype A (T155, T171, T255) and three genotype G (T159, T263, T67) were consistent with results obtained in the *gag* region. In contrast, the samples with gag-based HIV-1 genotype D (T164) and H (T274) gave sequences homologous to env-based genotype A and D, respectively. From the sample with the suspected dual infection with HIV-1 of genotypes A and G (T52), only genotype A–related *env* sequences were found in 20 analyzed clones.

DISCUSSION

The aim of this study was to determine the prevalent HIV-1 genotypes in patients with AIDS diagnosed in Lomé, Togo. More than twice as many males as females were present in the sample (Table I). The considerable excess of male patients is unusual in Africa, and was probably caused by a recruitment bias in the private outpatient clinics where patients were examined. The two genders were similar in age. The severity of clinical presentation was less than is usually observed in patients diagnosed in hospitals in Africa, again probably related to the fact that this was a population of outpatients.

To investigate the genomic diversity of HIV in Togolese patients, RT-PCR and direct sequencing of part of the *gag* region were performed. The primer regions used are relatively conserved among all characterized HIV strains, increasing the probability of successful amplification of more divergent strains. Different nucleotide signals were occasionally detected at a given position during sequencing, reflecting the multiple HIV-1 variants presumably contained within each RT-PCR product. In such instances, the nucleotide corresponding to the dominant signal was chosen, and this may have excluded minor HIV-1 variants from the analysis. The sequences derived therefore represent the predominant HIV-1 sequence circulating within an individual.

Phylogenetic analysis of the nucleotide sequence of the complete *env* or *gag* gene has revealed the same pattern of branching order with both, showing viruses clustering in the same major lineages [Becker et al., 1995]. The single exception is HIV-1 genotype E, which has no distinct *gag* region and clusters with genotype A for *gag* [McCutchan et al., 1996]. In accordance with previous studies [Salminen et al., 1993], it was found that comparable results can be obtained in phylogenetic analysis using only a subfragment of the *gag* gene (Fig. 1).

Phylogenetic analysis of 60 partial *gag* sequences identified the presence of four distinct HIV-1 genotypes in Togo (Fig. 2). The majority of isolates in this study belonged to genotype A (80%), with genotype G found in 16.7% of the patients. In three patients identified as genotype A and three as genotype G for *gag,* the genotyping was confirmed with the sequence obtained from the *env* region. Genotypes D and H were each found once. No HIV-1 genotype B, HIV-1 group O, or HIV-2 infections were found in this study. Sequences

belonging to the A genotype grouped in several clusters. A majority of these sequences were related to isolates found in countries from the same region, such as Ivory Coast [Louwagie et al., 1993] and Nigeria [Howard and Rasheed, 1996]. However, approximately one-third of the genotype A sequences in our patient population were more related to isolates found in Central and East Africa [Louwagie et al., 1993]. The fact that a majority of sequences clustered with other Togolese samples suggests that the HIV epidemic started with a small number of imported isolates.

Genotype G isolates have been identified in the Central African countries of Gabon [Janssens et al., 1994a; Delaporte et al., 1996], Central African Republic (CAR) [Murphy et al., 1993], Zaire [Potts et al., 1993], and Nigeria [Abimiku et al., 1994]. Its detection in this study and in a patient from Mali [Simon et al., 1996] would suggest that it is spreading to the more western countries of Africa. Genotype H has been identified in central African countries such as Gabon [Janssens et al., 1994a], Cameroon [Nkengasong et al., 1994], Zaire [Janssens et al., 1994a], and CAR [Murphy et al., 1993]. However, to our knowledge, it has not been previously reported in west Africa. Genotype D is most prevalent in the eastern countries of Africa [World Health Organization, 1994; Poss et al., 1997]. It is not unusual to detect occasional genotype D infections in the countries of central and west Africa [Delaporte et al., 1996; Takehisa et al., 1997], hence the detection of this genotype in Togo was not unexpected.

Dual infections with HIV-1 and HIV-2 have been previously described [Peeters et al., 1992]. Several groups have reported dual infections with HIV-1 and HIV-1 group O in Benin [Heyndrickx et al., 1996a], or with other, more closely related, genotypes of HIV-1 [Artenstein et al., 1995; Xin et al., 1995; Janini et al., 1996]. Coinfection by genotypes B and E was found in three Thai patients, and with genotypes F and D in one patient from Brazil. Although patients in these studies were not systematically investigated for infection by multiple HIV strains, such events appear to be rare (less than 5%) [Peeters et al., 1992; Heyndrickx et al., 1996a; Janini et al., 1996]. Whether infection with separate viral strains occurs simultaneously or sequentially has not yet been elucidated. In the case of patient T52 (Fig. 3), a 30-year-old male, coinfection with two of the most prevalent HIV-1 genotypes in the country is not surprising.

The suspected dual infection in sample T52 was not substantiated by analysis of the *env* region. This could be due to a recombination event between genotypes A and G [Robertson et al., 1995] or, alternatively, selective amplification of the A *env* region by the consensus primers. The more trivial explanation of contamination during amplification is less likely because of the care taken during the preparation of the sample, as well as the reproducibility of the amplification of A and G *gag* region sequences.

In patient T139, the clustering of the sequences with and without the 9-bp insertion suggests that both variants are derived from the same primary infection rather than from two separate infections (Fig. 3). It is likely that this mutation occurred during viral replication. It is also noted that the sequence obtained from patient T139 belongs to a cluster of 11 closely related sequences of HIV-1 genotype A, which are distant from published sequences of genotype A from other parts of Africa.

The apparent discrepancy between the *gag* and *env* regions of the D and H genotypes could indicate that they represent recombinant HIV genomes that, especially between A and D, are reportedly quite common [Robertson et al., 1995; Cornelissen et al., 1996; Brennan et al., 1997]. While sequence information on genotype H is limited, recombinants of this genotype have been described [Robertson et al., 1995]. The final resolution of this matter will, however, await sequence analyses of the complete genome of these isolates.

The genetic diversity of HIV-1 in Africa is known to be substantial [Janssens et al., 1997]. The distribution of HIV genotype observed in Togo is comparable to that observed in the neighboring countries of Benin [Heyndrickx et al., 1996b] and Ghana [Ishikawa et al., 1996], where genotype A predominates, as is the case throughout most of sub-Saharan Africa, including Togo.

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